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The Blue Coloration of the Common Surgeonfish, *Paracanthurus hepatus*——I. Morphological Features of Chromatophores

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ABSTRACT—In order to elucidate the mechanism by which the characteristic blue hues of the common surgeonfish, *Paracanthurus hepatus* (Acanthuridae), are generated, a histological and fine-structural examination was made on the integument. Under the epidermis of the sky blue portions of the skin, round iridophores without dendritic processes were compactly arranged in a double layer, which was lined by a single layer of melanophores. The configuration of each iridophore was very similar to that of the iridophores in the dermis of brilliantly blue-colored damselfish (Pomacentridae). In the cytoplasm, stacks of very thin light-reflecting platelets were arranged with their axes disposed radially from the apical part of the cell. In the dark blue region, by contrast, iridophores were found only rarely. These results indicate that the characteristic sky blue of the species may be dependent on the organized and double-layered arrangement of such light-reflecting cells. It seems likely that a multilayered thin-film interference phenomenon of the non-ideal type, occurring in the stacks of reflecting platelets, is primarily responsible for the generation of the blue hues. Iridophores of this type in the surgeonfish are the first to be found in a species that belongs to a family other than Pomacentridae.

INTRODUCTION

Many tropical fishes display beautiful colors and patterns, and the brilliantly bluish tints of some coral-reef fishes have attracted our particular attention. During the past decade, we have tried to understand the mechanism of the bluish colorations of some damselfish, including the blue damselfish, *Chrysiptera cyanea* [8, 15], and the blue-green damselfish, *Chromis viridis* [5]. To date, a fair amount of information has accumulated on the physics as well as the biology of the iridophores of these pomacentrids. Just under the epidermis of these fish, small, round or somewhat ellipsoidal iridophores can be found to be densely arranged in a single layer that resembles a brick pavement. Each cell contains a nucleus located in its apical part, from which a number of piles of light-reflecting platelets are disposed radially in the cytoplasm. Fine-structural observations have revealed that the reflecting platelets are not more than 5 nm thick. Some of these cells are motile, while some others are the immotile. The motile cells are believed to be responsible for the remarkable color changes in color that are characteristic of these fishes.

Among the beautiful fish found around coral reefs, the common surgeonfish or the regal tang, *Paracanthurus hepatus*, is also well known for its beautiful two-tone bluish colors, brighter and deeper, distributed over its trunk. We recognized, however, that, although the hues can be described as blue in a broad sense, they are rather different from the fluorescent-like blue displayed by many damselfishes. Having rather low purity, the lighter hue of the surgeonfish may appropriately be called "sky blue" or "cerulean blue", while the dark part appears "dark blue" or "midnight blue". In the present work, therefore, investigations were made to identify the morphological basis for the characteristic bluish hues displayed by this acanthurid fish.

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MATERIALS AND METHODS

Material

The material used was the common surgeonfish, *Paracanthurus hepatus*, which belongs to the Acanthuridae (suborder Acanthuroidei, order Perciformes). They are described as being rather common around coral reefs in Indo-Pacific waters. Young adult forms with body lengths between 40 and 60 mm were purchased from local dealers in Tokyo and in Chiba Prefecture, and they were maintained in a seawater aquarium at our facility prior to sacrifice.

Light and electron microscopic observations

First, the general morphology of the skin was studied under the light microscope. Decapitation of a fish was rapidly followed by immersion of the trunk in physiological saline solution which had the following composition (mM): NaCl (128), KCl (2.7), $CaCl_2$ (1.8), (*R*)-(+)-glucose (5.6), and Tris-HCl buffer (10.0; pH 7.2). Then the body was fixed in a solution of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2). During the fixation for 2 hr at room temperature, the sky blue and/or dark blue region of the skin was carefully excised and cut into small pieces for better penetration of the fixative. After rinsing with 0.1 M phosphate buffer (pH 7.2), these pieces were dehydrated in a graded ethanol series. The samples were embedded in 2-hydroxyethyl methacrylate (Quetol 523M; Nisshin EM, Tokyo). One-μm sections were cut on a Porter-Blum MT-1 ultramicrotome (Ivan Sorvall,

Newtown, CT) with glass knives. After they had been spread on glass slides, the sections were stained with toluidine blue for general histological examination of the skin, or they were treated with van Gieson's stain that is specific for collagenous components.

For fine-structural observations, a fixative composed of 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1M phosphate buffer (pH 7.2) was employed. While it was immersed in the fixative for a total of 2 hr at room temperature, the trunk was first filleted along the vertebral column. Then, the sky blue or dark blue region of the skin was carefully excised and cut into small squares of about 1 mm^2. Pieces from the yellow part of the tail fin were sometimes fixed for fine-structural observations of the integument. After a 30-min wash with 0.1M phosphate buffer, the specimens were postfixed in a solution of 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) for 30 min at 4°C. These samples were then dehydrated in a graded ethanol series, treated with methyl glycidyl ether, and embedded in Epoxy resin (Quetol 812; Nisshin EM).

Ultrathin sections were cut on the MT-1 ultramicrotome with a diamond knife and then mounted on Formvar™-coated grids. Specimens of the trunk skin were cut vertically to the plane of the skin. When a piece of tail-fin was used, the inter-ray membrane between adjacent fin-rays was cut perpendicularly to the direction of the rays. All the sections were stained with 3% uranyl acetate for 15 min and then with Reynolds' lead citrate for 7 min, and they were viewed with an electron microscope (JEM-1210, JEOL, Tokyo) operated at 80 kV.

RESULTS

Preliminary observations

Figure 1 shows a photograph of a live young specimen of the present material, namely, the common surgeonfish, *Paracanthurus hepatus*, hovering in a small aquarium that contained seawater. The sky blue background coloration that extended over the greater part of the trunk is characteristic of this species, and the region near the middle part of the trunk, namely, the area between the two "handles" of the dark scissors pattern, was used for most of our observations (SB in Fig. 1). The dark blue scissors-like pattern is also a prominent feature of this species, and pieces of skin from this region (DB in Fig. 1) were also used for comparisons. The characteristic pattern on the skin was still clearly visible in the fixative after decapitation. Thus, it was rather easy to prepare specimens from the designated portions of the skin for morphological examination.

The composition of chromatophores in the skin of the yellow portion of the caudal fin (Y in Fig. 1) was also examined for further comparisons.

Histological observations

The photomicrographs in panels A and B of Figure 2 show parts of 1-μm sections that were cut vertically across the integument of the sky blue part of a common surgeonfish which had a body length of about 50 mm. The section displayed in panel A was stained with toluidine blue to show the general construction of the skin, whereas that in panel B was treated with van Gieson's stain to demonstrate collagenous components in the tissue. The epidermis of a fish with a body length of about 50 mm was about 100 μm thick or slightly more. Near the surface of the epidermis, a number of mucous and serous cells were visible. No epidermal chromatophores were found at any level. Just under the epidermis, but above the thick collagenous layer, there were layers of chromatophores: a rather homogeneous lucent zone was lined with a very dense or black sheet underneath. The lucent zone was composed of polyhedral iridophores without dendritic processes, while the latter was a sheet of melanophores. Closer investigations indicated that the iridophores were densely packed in a double layer. The melanophores of the present material assumed a configuration that resembled the configuration found in amphibians [1, 2] or bluish damselfish [5, 8, 15]: the melanophores extended their dendritic processes upwards into the spaces among the iridophores, frequently reaching the boundary between the dermis and the epidermis.

Under the layer of chromatophores, a thick, homogeneous layer of uniform thickness was visible. When the histological sections were treated with van Gieson's stain, this layer stained red (Fig. 2B), a result that suggests the collagenous nature of this material.

In the present material, the main bodies of scales or scutes were buried in the dermis, just beneath the layer of chromatophores (Fig. 2B). They were also heavily stained with van Gieson's stain. Although not included in the present photomicrographs, a few denticles were normally found to protrude from these scales beyond the epidermis. Both the scales and the thick collagenous layer should contribute to the robustness of the integument, a characteristic feature of fish in the Acanthuridae.

Fine-structural observations

Figure 3 shows an electron micrograph, taken at low magnification, in which, just under the epidermis, a double layer of iridophores is clearly visible. Thus, the iridophores can be categorized into two groups, namely, those located in the upper layer and those located in the lower layer. The double layer of iridophores was lined with a monolayered network of dermal melanophores. Being classified as loose connective tissue, the tissue that included the melanophores contained various dermal components, such as bundles of collagen fibrils, blood capillaries and nerve fibers. Processes of melanophores were frequently observed to invade the double layer of iridophores (Fig. 4). Sometimes, these processes were accompanied by bundles of collagen fibrils that ran parallel to the processes (Fig. 4). Occasionally, the processes of melanophores were found to reach the basal lamina that lined the epidermis. However, the perikarya of the melanophores were always found below the double layer of iridophores.

The general morphology of the iridophores was fundamentally similar to that of the iridophores of the damselfish species studied to date [5, 8, 15], with stacks of very thin light-reflecting platelets in the cytoplasm. The stacks were arranged so that their axes radiated from the apical pole of

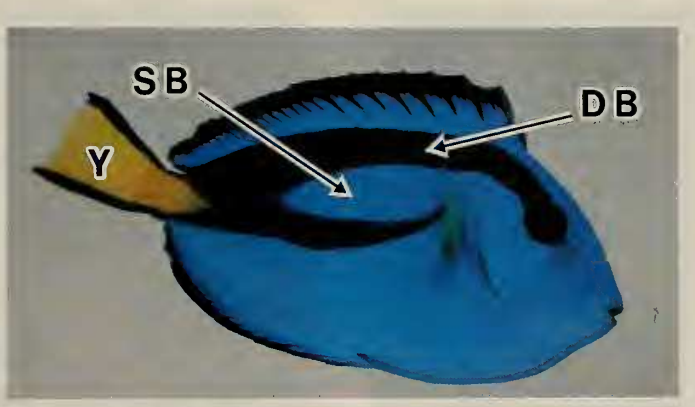

FIG. 1. Photograph of a young surgeonfish, *Paracanthurus hepatus*. The body length of this individual was about 45 mm. The sky blue region in the middle part of the trunk (SB), the dark blue region dorsal to the former (DB) and the yellow portion of the tail fin (Y) were examined.

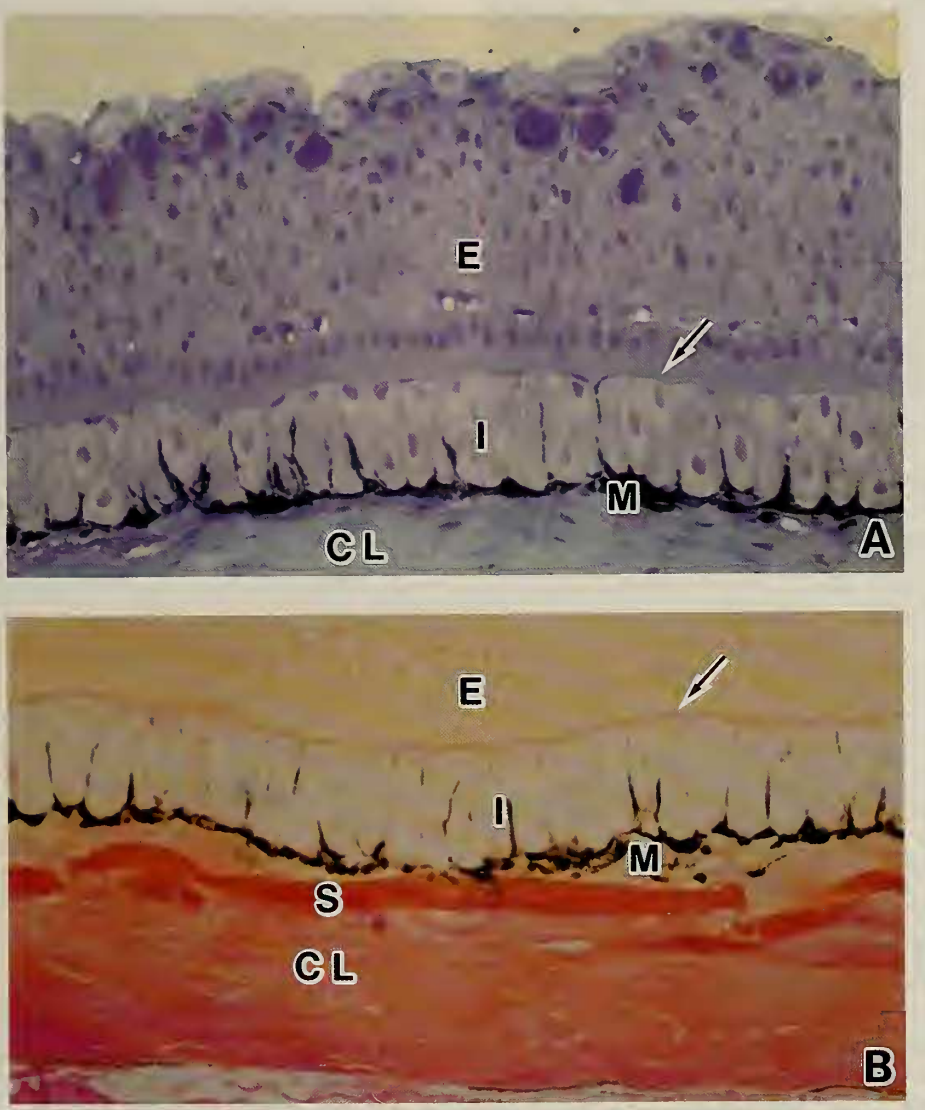

FIG. 2. Photomicrographs of 1-μm sections cut vertically across the integument of the sky blue part (SB in Fig. 1) of a surgeonfish with a body length of about 50 mm. **A**: Stained with toluidine blue. **B**: Stained with van Gieson's solution. Arrows indicate dermo-epidermal junction. CL, collagenous layer; E, epidermis; I, iridophore; M, melanophore; S, scale. $\times$360.

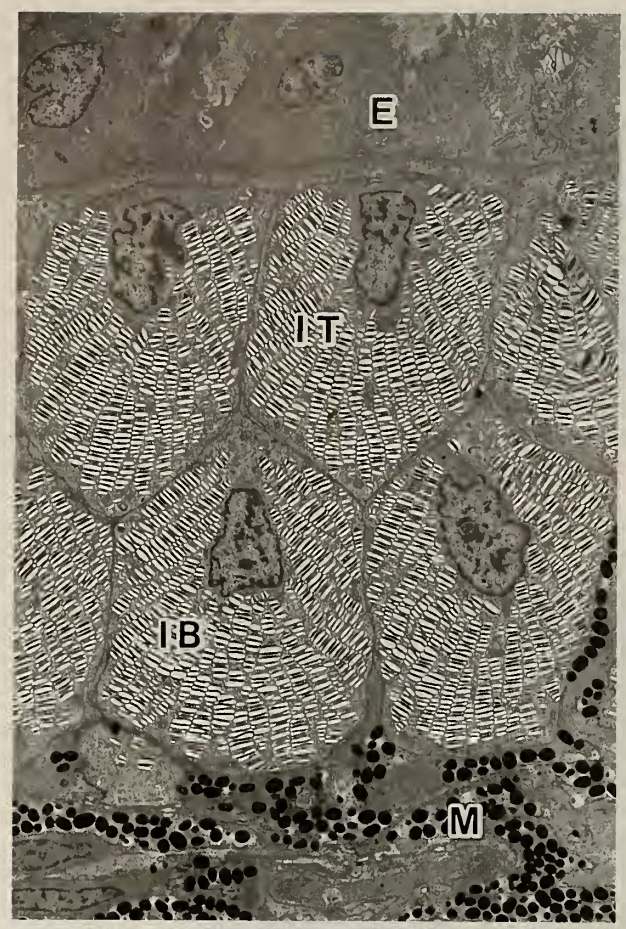

Fig. 3. Low-power electron micrograph of a vertical section across the sky blue part of the dermis of a surgeonfish. Iridophores constitute a double layer, which is lined by a sheet of melanophores. In the iridophores in the upper layer, nuclei are present in the topmost regions, while in iridophores in the bottom layer, nuclei are located more centrally. E, epidermis; IB, iridophore of the bottom layer; IT, iridophore of the top stratum; M, melanophore. ×3,700.

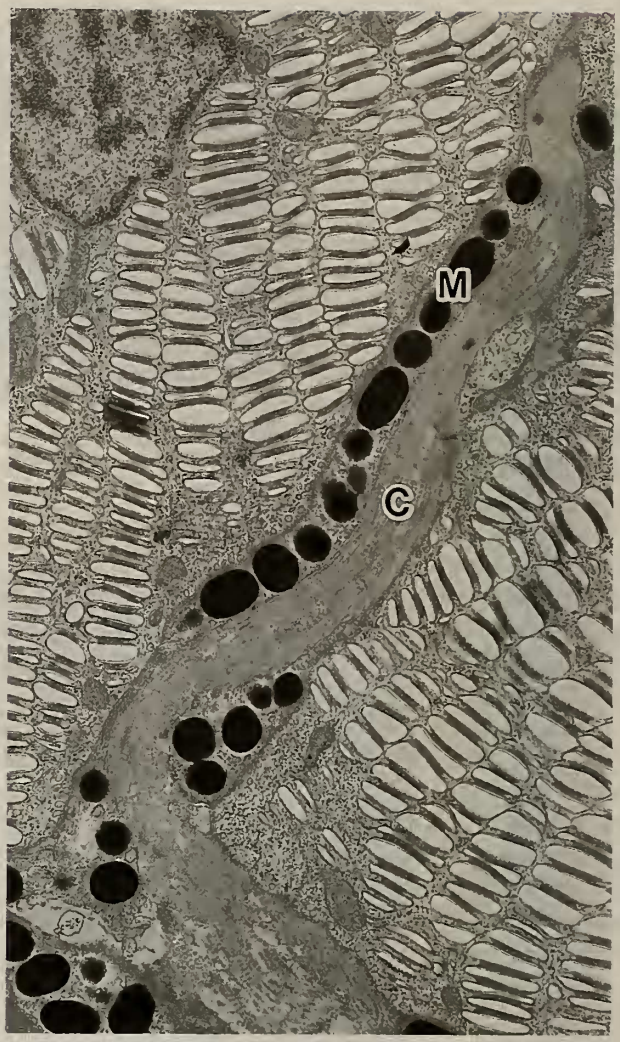

Fig. 4. Electron micrograph of part of the double layer of iridophores, showing that a process of a melanophore is invading the space between two iridophores. A bundle of collagen fibrils is seen to run parallel to the process. C, bundle of collagen fibrils; M, process of a melanophore. ×10,600.

the cell.

We noted a clear difference in the location of the nucleus between the two types of iridophore of the surgeonfish. In the iridophores in the top layer of the double layer, the nucleus was always found in an extremely apical region of the cytoplasm (Figs. 3 and 5A). By contrast, nuclei in the iridophores in the lower layer were more or less in the center of the cells (Figs. 3and5B). In each case, the arrangement of the stacks of reflecting platelets was analogous, as described above.

Each reflecting platelet was found to be enveloped by a smooth membrane, which may possibly have been the cisternal membrane of the smooth-surfaced endoplasmic reticulum (Fig. 6). The platelets seemed to be very uniform in thickness. However, it was not so easy to estimate their thickness exactly on electron micrographs, because of their extreme thinness. It may safely be said, however, that platelets were not more than 8 nm thick.

Electron-dense material was normally detectable between the adjacent cisternae that contained platelets. Careful observations indicated that such material was aggregated around the central region of the platelets, suggesting that the material might serve as a spacer to keep the distance between contiguous platelets both uniform and constant.

We also often observed that the reflecting platelets in pairs of adjacent piles were regularly interdigitated (Fig. 6). Presumably, such architecture is also useful for maintaining similar spacing between contiguous platelets in a particular pile as well as in adjacent piles. Such an interdigitated arrangement has not been observed in iridophores of the other fish species studied to date.

Figure 7 shows part of the cytoplasm of an iridophore, in which the plane of the reflecting platelets was almost parallel to the plane of the section. Some reflecting platelets clearly show a hexagonal crystalline pattern.

The very dark bluish portions of the skin, indicated as

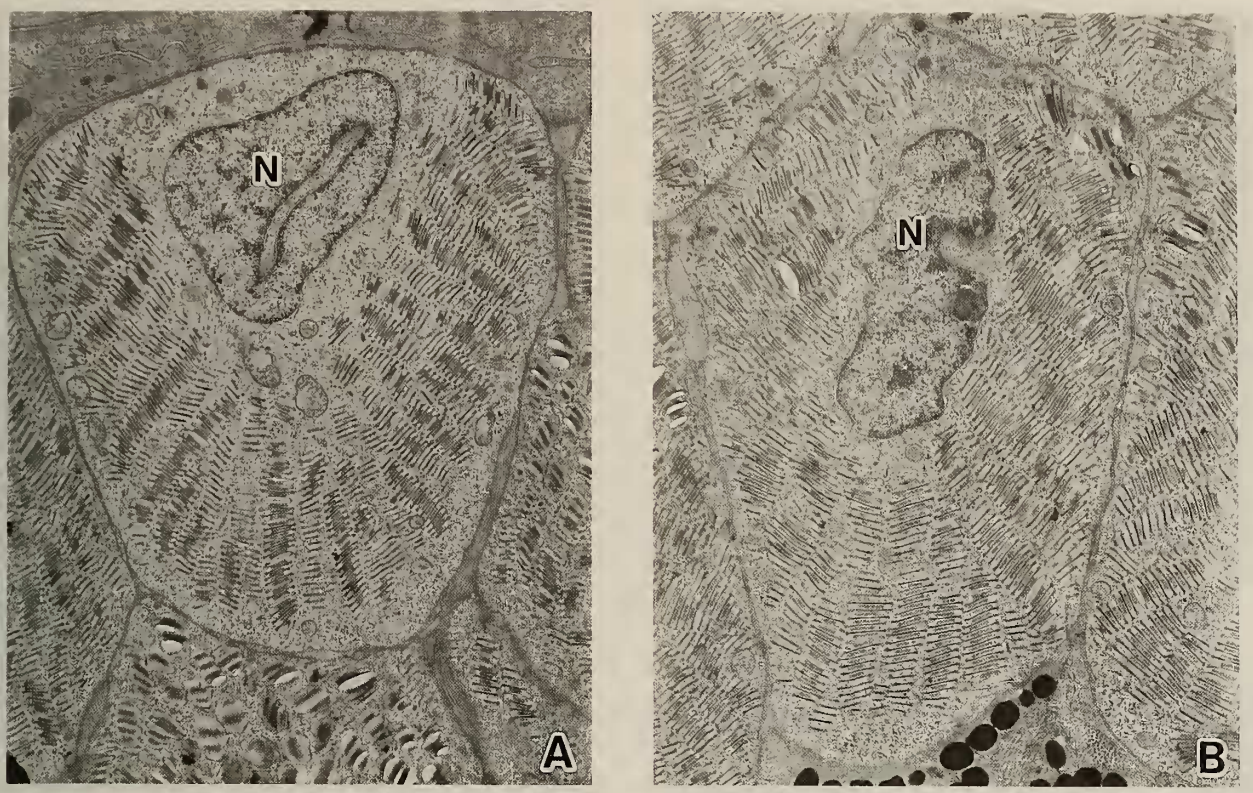

FIG. 5. Electron micrographs of iridophores that form a double layer in the dermis. A: An iridophore from the upper layer. The nucleus is located in the apical part of the cell. Stacks of reflecting platelets are arranged radially from the region of the nucleus. There are no stacks of platelets between the nucleus and the apical part of the cell membrane. Invagination of the nuclear envelope is seen as a long streak. **B**: An iridophore from the bottom layer of iridophores. The nucleus is located more centrally than in the cell in A (cf. Figs. 3 and 4). Stacks of reflecting platelets are visible in the apical cytoplasm. N, nucleus. ×7,800.

FIG. 6. Electron micrograph of part of the cytoplasm of an iridophore, showing details of the arrangement of the stacks of platelets. Arrows indicate reflecting platelets, while arrowheads indicate cisternal membranes that enclose the platelets. The reflecting platelets in adjacent stacks are frequently interdigitated in a very regular manner. DM, dense material. ×43,000.

FIG. 7. Electron micrograph of part of the cytoplasm of an iridophore. The section was cut, by chance, almost parallel to the plane of the reflecting platelets. In some reflecting platelets, a hexagonal crystalline pattern is visible. ×40,000.

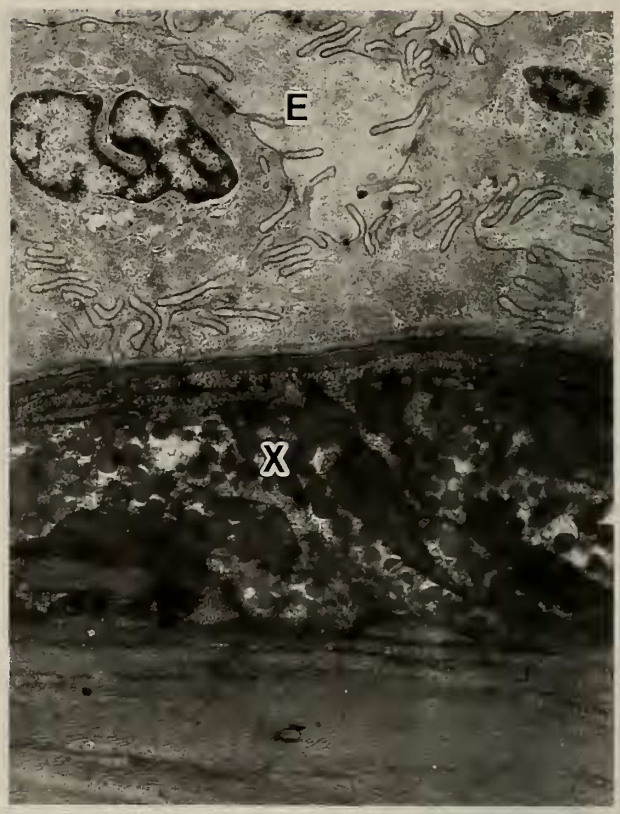

FIG. 9. Electron micrograph of part of the skin between two fin-rays of the caudal fin. The section was cut vertically with respect to the plane of the skin and also to the fin-rays. Under the epidermis, part of the cytoplasm of a xanthophore containing a number of yellow pigment granules, the xanthosomes, is seen. E, epidermis; X, xanthophore. ×7,500.

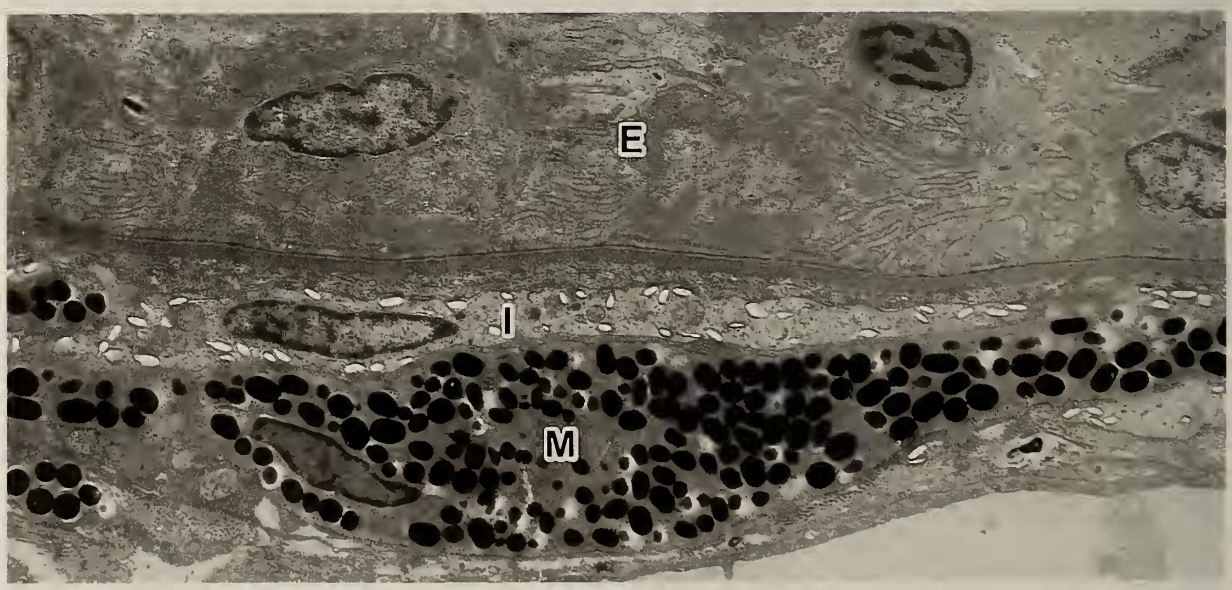

FIG. 8. Electron micrograph of a vertical section through skin taken from the dark blue region, indicated as DB in Fig. 1. A thin layer of cytoplasm of an iridophore in monolayer is seen just above the layer of melanophores. Being variously oriented, small light-reflecting platelets are found scattered in the cytoplasm. A monolayer of the cytoplasm of a melanophore lines the iridophore. E, epidermis; I, iridophore; M, melanophore. ×7,500.

DB in Figure 1, were also examined mainly with regard to the composition of chromatophores. Under the epidermis, we were always able to find a well defined sheet of melanophores (Fig. 8). By contrast, iridophores were only sparsely scattered, and in no case did they form a double layer of the type observed in the sky blue part of the skin. The iridophores themselves were also very thin. The disposition of the reflecting platelets in the cells seemed to be rather irregular, as compared with that in the iridophores in the sky blue region. Stacks of platelets were much smaller, and many of the platelets were rather randomly distributed.

The bright yellowish skin of the caudal fin was examined last of all. Xanthophores in the dermis, containing a number of yellow pigment granules (xanthosomes), were the only chromatophores found (Fig. 9). Such xanthophores can be considered to be responsible for the bright yellow coloration of this region of the skin.

DISCUSSION

The structural organization of the integumentary tissues of the present species, the common surgeonfish, was fundamentally similar to that described in teleosts, which are phylogenetically not very distant from the present material [16, 17]. In the present material, the scales were not recognizable from the outside. However, buried in the dermis, numerous small scales or scutes were found from which denticles or spines protruded through the surface of the skin *via* the layer of dermal chromatophores and the epidermis. These denticles are responsible for the rough feel of the surface of the skin. The dermal collagenous layer below the double layer chromatophores was very well developed. Apparently, both the sheet of scutes and the collagenous layer contribute to the robustness of the skin of this species. The epidermis was also found to be fairly thick, as compared to that of other fishes of similar size.

The nature of the chromatophores and their disposition in the skin are primarily responsible for the coloration of an animal. Such a statement should also apply to the present species, which, with its beautiful colors, is very popular among aquarists. Indeed, such bright blue hues are found only infrequently, even in teleosts. However, since vertebrates do not have blue chromatophores or blue pigment in the tissues, we have to seek the origin of bluish tints in other structures in the skin.

In the surgeonfish, no chromatophores were found in the epidermis. Thus, the characteristic hues of the skin must be dependent primarily on the chromatophores in the dermis. In the present study, we found a very particular combination of chromatophores in the dermis, with a rather similar organization to that found in the dermis of blue-colored damselfish [5, 8, 15]. Thus, it is not unreasonable to suppose that similar optical phenomena may be involved in the generation of bluish coloration in the surgeonfish and the damselfish.

In amphibians and reptiles, dermal chromatophore units have been shown to be responsible for the production of a characteristic greenish tone [1, 2]. The organized, layered arrangement of chromatophores, namely, the xanthophores, iridophores and melanophores, from top to bottom in the dermis, is the architectural requirement for such greenish coloration. Nishioka and Ueda [14] further showed that the dermal chromatophore units of blue mutant frogs (*Rhacophorus schlegelii*) lacked the xanthophores that are normally present at the top of these units. Namely, simpler units consisting of the light-scattering iridophores and the dark sheet of melanophores that underlies the iridophores are responsible for the production of the bluish tone of such mutants. In normal green frogs, the overlying xanthophores function as a yellow filter to shift the spectral reflectance peak towards a region of longer wavelengths, namely, green, by eliminating light of shorter wavelengths. In these cases, the scattering of light in the iridophores has been explained as the Tyndall phenomenon, associated with Rayleigh scattering, by which light of shorter wavelengths is more effectively scattered [1, 2].

During the evolution of teleosts, the three-layered chromatophore units of the type found in greenish amphibians and reptiles failed to develop [4]. As mentioned above, simpler bilayered units composed of iridophores and melanophores are found in the dermis of bluish damselfish [5, 8, 15]. The construction resembles that in the blue mutants of amphibians. However, in the iridophores of these pomacentrids, the light-reflecting platelets are arranged in a strikingly organized manner in the cytoplasm, and light has been shown to be reflected by a multilayered interference phenomenon of the non-ideal type, rather than by Tyndall scattering [4, 10].

Although included in the same very large order, Perciformes, fish of the family Acanthuridae (suborder Acanthuroidei), to which the present material belongs, and those of Pomacentridae (suborder Percoidei), of which all damselfish are members, are phylogenetically rather distant from each other. We were, therefore, surprised to find that the morphology of the iridophores of the present material so closely resembled that of iridophores of bluish damselfish [4, 5, 8, 15]. Both surgeonfish and the damselfish possess iridophores of a very similar that cause their bluish tints.

The currently available information about iridophores indicates that those of teleosts can be divided into several classes [4]. Containing few stacks of thick reflecting platelets, iridophores of the first type are known to function in the generation of the silvery glitter, or very strong whiteness of lateral or belly skin, acting *via* a multi-layered thin-film interference phenomenon of the ideal type [3, 4, 9, 10]. These cells are believed to be immotile. Also containing only a few stacks of large, very thin platelets, iridophores of the lateral blue-green stripe of neon tetras reflect light within a limited region of the spectrum by thin-film interference of the non-ideal type [11, 13]. These platelets are categorized as a type of motile iridophore, and the spacing between the platelets varies in response to various environ-

mental cues, leading to changes in spectral reflectance. The iridophores of bluish damselfishes have already been described above. In many cases, they are motile, and, as a result, the skin that contains these iridophores can vary its hue [5, 8, 15]. Iridophores of the last type are dendritic cells in which tiny platelets can move centripetally and centrifugally, resembling other common dendritic chromatophores such as melanophores, erythrophores and xanthophores [6, 7]. The iridophores of the present material are clearly of the damselfish type, although cellular motility is not significant, if it existed at all (Goda *et al.*, in preparation).

From our observations and experiments, we had previously reached the conclusion that, in bluish damselfishes, the iridophores have a definite role in the generation of the characteristic hue. It is, therefore, quite plausible to assume that the iridophores described in this study play the same role in the surgeonfish. In the surgeonfish also, a multiple layered thin-film interference phenomenon should be primarily responsible for the reflection of shorter-wavelength light. The light-absorbing black sheet containing large amounts of melanin functions to absorb light that has passed through the layer of iridophores, being responsible for the purer bluish tint of the skin.

It should be noted, however, that the mode of disposition of the iridophores in the dermis is not entirely the same in the two types of fish. In the dermis of damselfish, the iridophores were found without exception in a monolayer, but those in the surgeonfish were arranged as a double layer. This difference may be the principal cause of the difference in coloration between the surgeonfish and the damselfish.

In the iridophore of both the surgeonfish and damselfish, a number of piles of very thin light-reflecting platelets are arranged radially from the apical part of each cell. Thus, the axes of piles are oriented at various angles to the surface of the skin. Incident light is maximally reflected along the axis as a result of a multilayered thin-film interference phenomenon, as mentioned above. In the bluish damselfish, the light-reflecting platelets constituting a pile are extremely thin, each one being not more than 5 nm thick. Such architecture favors the reflection of light with a sharp spectral peak and, indeed, a fluorescent-like hue of very high purity is produced [4]. In the surgeonfish, by contrast, the platelets seemed to be a little thicker than those of the damselfish, each being 7–8 nm thick. It is known that, in multilayered interference systems, the spectral reflectance peak becomes sharper when the individual platelets in a stack are thinner. As if mixed with a larger amount of white paint, the sky blue of the surgeonfish is less pure or has lower "chroma", in terms of the Munsell color notation system, than the brilliant cobalt blue of the blue damselfish.

The iridophores are present in a double layer in the surgeonfish. Such a feature should also be related to the production of sky blue coloration rather than the very pure cobalt blue expressed by the reef damselfishes. When iridophores are in a single layer, the interference due to light reflected from a pile of platelets is not disturbed. However, it seems plausible that the light reflected from the iridophores in the bottom layer is complicatedly scattered by the reflecting platelets in the iridophores in the upper layer. If the iridophores of the surgeonfish were present in a monolayer, the bluish tint would be purer or more fluorescent-like, resembling that of the damselfishes.

In the iridophores on the epidermal side, a nucleus was found in an extremely apical part of the cytoplasm, while in the iridophores in the bottom layer, each nucleus of was in the center of the cell. We cannot explain this difference at present, because, in both cases, the arrangement of the piles of reflecting platelets in the cells was practically the same.

The fact that the epidermis is rather thick, as compared with that of other fish of comparable size, may also be related to the lower purity of the integumentary coloration of the present species. Light-scattering components in the epidermis may also contribute to widening of the spectral peak and elevation of the base line of the spectrum. However, the epidermis of fish is usually more transparent than that of terrestrial tetrapods.

In many fish with relatively thick epidermis, epidermal melanophores can be found [4, 12], but we failed to find such cells in the present material. The absence of pigmentary material in the epidermis should be advantageous for the more effective displaying of a bright hue because, if such cells were present in overlying structures, the light reflected from the iridophores in the dermis would be disturbed and weakened.

In the present analysis, we found for the first time iridophores, with a configuration very similar to that of iridophores of coral-reef bluish damselfish in a species that belongs to a family other than Pomacentridae. A more extensive survey of acanthurid species may result in the finding of such iridophores among species that are closely related to the present species. It is also possible that such iridophores will be found in other families or orders of teleosts. Such surveys would surely provide useful clues as to whether such iridophores have a common origin or have evolved sporadically in various groups of fish in more recent evolutionary time. Thus, the present results may provide some initial clues to the taxonomy of fishes within the order Perciformes.

A more detailed discussion of the optical mechanisms involved in the generation of the characteristic sky-blue and dark-blue portions of the strikingly beautiful surgeonfish will be presented in a separate paper (Goda *et al.*, in preparation).

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